RESEARCH PAPER

HPTLC Method Validation, GC-MS/MS Characterization and Anticancer Evaluation of Arisaema tortuosum Tubers against A431 Cells.

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ABSTRACT

Arisaema tortuosum (Wall.) Schott is an ethnomedicinally significant plant lacking comprehensive phytochemical profiling. This study characterized tuber extracts prepared by sequential solvent extraction, with yields varying by polarity: chloroform (2.5%), ethyl acetate (2.9%), ethanol (6.3%), and aqueous (11.2%) w/w. Preliminary screening confirmed alkaloids, flavonoids, phenolics, and tannins. Ethanol extract showed superior total phenolic (0.878 mg Gallic Acid Equivalent/100 mg) and flavonoid (0.805 mg Quercetin Equivalent/100 mg). A validated HPTLC method was developed (ICH-compliant) which quantified lupeol with high linearity (R^2 = 0.999, LOQ 3.70 µg/spot), precision (intra-day %CV 0.67–1.03), accuracy (99.92–100.05% recovery), and sensitivity (LOD 1.22 µg/spot) content. Lupeol (Rf 0.67) was highest in ethanol extract (0.667% w/w), confirmed by HPTLC profiling and further characterized by GC-MS/MS analysis by matching retention times (sample: 31.85 min, standard: 31.86 min) and characteristic molecular ion (m/z 426). Cytotoxicity against A431 human epidermoid carcinoma cells revealed potent activity for the ethanol extract (IC_{50} 13.49 µg/mL), approximately 12.5-fold stronger than lupeol standard (IC_{50} 168 µg/mL). The ethanol extract of A. tortuosum is a promising anticancer lead, supported by a robust phytochemical profile and a validated HPTLC method for quality control.

Keywords: GC-MS/MS, HPTLC, ICH guideline, Method Development and Validation, NRU assay, A431 Cell Line.

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1. INTRODUCTION

The enduring value of plant-based natural products in drug discovery is well established, serving as an essential source for a diverse range of bioactive compounds that provide templates for new therapeutics [1]. This is particularly relevant for species within the *Arisaema* genus, which are gaining scientific recognition as potential sources of immunity-boosting compounds, transitioning from botanical curiosities to subjects of serious pharmacological inquiry. [2]. This focus forms part of a broader renaissance in herbal medicine, where a renewed appreciation for traditional knowledge systems is driving researchers to apply advanced tools to validate ancient wisdom [3]. A prime candidate for this approach is *Arisaema tortuosum*, a plant whose leaf extracts demonstrate a rich antioxidant profile, providing a molecular basis for its historical use in traditional healing practices [4].

However, translating traditional promise into evidence-based therapy requires unwavering scientific rigor, beginning with the fundamental principle of standardization to ensure consistent and reproducible plant material [5]. This process involves creating a unique chemical fingerprint, for which techniques like High Performance Thin Layer Chromatography (HPTLC) are essential, confirming botanical identity and ensuring chemical consistency across batches [6]. Such foundational work enables significant discoveries, as demonstrated by the recent isolation of anticancer molecules from *Arisaema flavum*, a close relative, signaling the genus's potential for yielding novel therapeutic entities [7].

To fully contextualize *A. tortuosum*, its deep-rooted ethnobotanical legacy must be considered; comprehensive reviews document its traditional use against conditions ranging from inflammation to tumors, providing a historical roadmap for modern research [8]. Building on this, systematic reviews confirm the genus's chemical diversity and broad bioactivity, strengthening the scientific rationale for focused investigation on specific species like *A. tortuosum* [9]. Further supporting its pharmacological value, research reveals that the plant's utility is not confined to a single domain, with leaf extracts showing significant antiviral activity and thus revealing a broader defensive capability [10]. Additionally, the identification of nutritional metabolites

in some *Arisaema* species suggests a dual role that complements targeted therapy with foundational health support [11].

Empirical validation extends to its antibacterial properties, where laboratory studies using bacterial growth kinetics have quantitatively confirmed its efficacy, translating traditional knowledge into modern scientific terms [12]. Beyond establishing *what* the plant does, understanding *how* it acts is crucial; investigations have begun to unravel its mechanism, identifying specific lectins that bind to cell surface sugars, thereby inhibiting cancer cell proliferation [13]. Discoveries of this nature elevate plant extracts from simple remedies to valuable lead compounds, which are essential starting points for developing optimized pharmaceutical agents [14]. The validation pathway often integrates traditional insight with modern technology, as seen in studies on *A. tortuosum* that combined computational prediction with laboratory work to confirm its antioxidant and specific anti-breast cancer activities [15].

Furthermore, the plant's pharmacological repertoire appears extensive, with related species demonstrating anxiolytic effects, indicating potential applications in neurological and emotional well-being [16]. The activity is not limited to isolated compounds; studies on crude tuber extracts have confirmed a combination of anti-proliferative, antioxidant, and anti- inflammatory activities, illustrating the therapeutic importance of the plant's complete chemical profile [17]. The foundational evidence for this research trajectory was established by a pioneering study that successfully isolated a lectin from *A. tortuosum* and demonstrated its direct anticancer activity in vitro, providing the first mechanistic evidence for its traditional use against tumors [18]. Building upon this sequentially established foundation, the present study aims to integrate these diverse threads ethnobotanical clues, chemical standardization, compound discovery, and mechanistic insight into a unified investigation: Our objective is a comprehensive phytochemical and biological profiling of *Arisaema tortuosum* tubers. This will begin with detailed chemical characterization using HPTLC and GC-MS to establish a complete phytochemical map. This map will then guide a systematic biological evaluation of the extract's cytotoxic potential against human cancer cell lines, thereby rigorously translating the traditional legacy of *Arisaema tortuosum* into a validated contribution to modern medicine.

2. MATERIAL AND METHOD

2.1 Reagents and Standards

All chemicals and solvents used were of high analytical grade and purchased from Merck (Darmstadt, Germany). From this, solution was analysed using Linomat applicator on TLC aluminum plates precoated with silica gel 60 F254 (10 · 10 cm, 0.2 mm thick) purchased from E. Merck Ltd. [17, 19, 20].

2.2 Plant material

Fresh tubers of Arisaema tortuosum (Wall.) Schott were harvested from the Raisen Region of Madhya Pradesh, India, in December 2022. The plant was verified by a Botanist Scientist (क्र./एम.एफ.पी/ब.पी./प.अ/2022/2421) at the Minor Forest Produce Processing and Research Centre in Bhopal, Madhya Pradesh (A PSU under Government of Madhya Pradesh, India).

3. EXPERIMENTAL

The complete experimental workflow is illustrated in Figure *1* Flowchart illustrating the sequential stages of the phytochemical research process. Tubers of *Arisaema tortuosum* were sequentially extracted using solvents of increasing polarity. The resultant extracts underwent preliminary phytochemical screening followed by spectrophotometric determination of total phenolic and flavonoid content. A high-performance thin-layer chromatography (HPTLC) method was developed and validated for lupeol quantification, with subsequent compound isolation assisted by an HPTLC Interface. The isolated lupeol was characterized using gas chromatography–tandem mass spectrometry (GC–MS/MS). Finally, the cytotoxic activity of the extracts and the isolated compound was evaluated in vitro against A-431 cell lines.

Figure 1 Flowchart illustrating the sequential stages of the phytochemical research process

3.1 Processing and Preparation of Plant Material

The tubers were thoroughly washed. The cleaning procedure was divided into the following manner: The decayed or deteriorated plant matter was removed first. The samples were then washed with tap and purified water. To extract excess water, the washed plant materials were covered with blotting paper. Plant material was then chopped and shade-dried shortly after washing Figure 2 . The primary goal of drying is to extract the water content of the plant for processing. The dried tuber was finely powdered using an electric grinder, sieved and stored in polyethylene bags [19, 21].

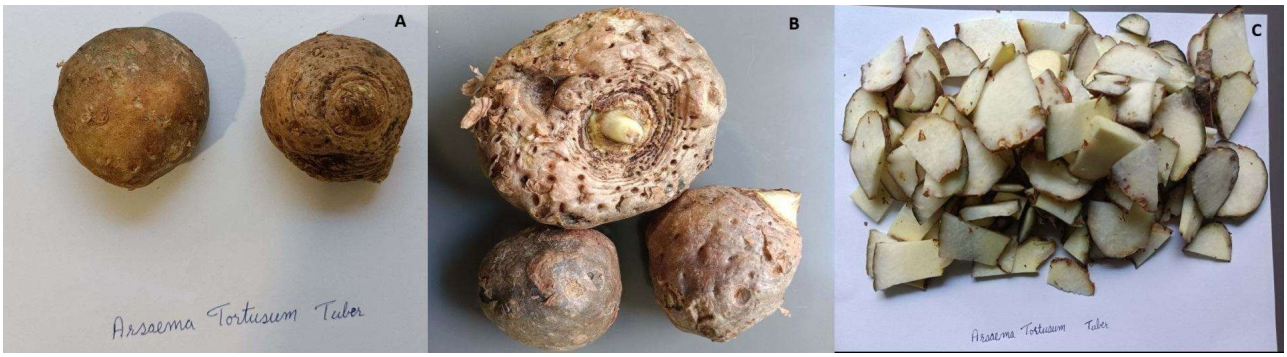

Figure 2 Sequential steps in the preparation of Arisaema tortuosum tubers: (A) Collection of tubers, (B) cleaning process, and (C) cutting the tubers into slices for experimental use.

3.2 Extraction by Maceration Process

The Dried powder (40 g) was extracted with different solvents (chloroform, ethyl acetate, ethanol and water) using the sequential maceration technique [22]. The samples were left for 48 hours under sterile environment. The liquid extract was then filtered through Whatman filter paper no. 40. The filtrate was kept in a water bath at 80-90ºC till the extract was dried.

3.3 Biochemical Assays

Preliminary phytochemical screening of all four extracts was conducted to evaluate the presence of various phytochemicals found in plants. The crude extracts were analysed for presence of secondary metabolites, such as alkaloids, phenolic compounds, flavonoids, saponins, tannins, and glycosides. [23, 24].

3.4 Quantitative Analysis of Phytoconstituents

3.4.1 Evaluation of Total Phenol Content

The total phenolic content was determined using the modified Folin Ciocalteu method. 10 mg of Gallic acid was dissolved in 10 ml of methanol, yielding concentration of 10 to 50 µg/ml. 10 mg of dried extract was solubilized in 10 ml of methanol and filtered. 2 ml (1 mg/ml) of this extract was used for the quantification of phenol. The extract and each standard (2 ml) were mixed with 1 ml of Folin Ciocalteu reagent (diluted with distilled water at a 1:10 v/v ratio) and 1 ml (7.5 g/l) of sodium carbonate. The mixture was vortexed for 15 seconds and allowed to stand for 10 minutes to facilitate colour development. Absorbance was taken at 765 nm with a spectrophotometer [19, 24, 25, 26].

3.4.2 Evaluation of Total Flavonoids Content

The total flavonoid content was evaluated utilizing the aluminium chloride method. 10 mg of Quercetin was dissolved in 10 ml of methanol, and several aliquots ranging from 5 to 25 µg/ml were produced in methanol. Ten milligrams of the desiccated extract was solubilized in 10 millilitres of methanol and filtered [19].

3 ml (1 mg/ml) of the extract was utilized for quantification of flavonoids. One millilitre of 2% AlCl3 solution was combined with three millilitres of extract or each standard and permitted to stand for 15 minutes at ambient temperature, and absorbance was recorded at 420 nm [25, 24, 27, 21]**.**

3.5 Preparation of plant extracts

To obtain bioactive chemicals from *Arisaema tortuosum* (AT), dried coarse plant material (300 mg) was separately dissolved in four different solvents: ethyl acetate, chloroform, ethanol and water. Each mixture was sonicated for 15 min to enhance solubility, followed by centrifugation at 3000 rpm for 10 minute to filter out the insoluble residue. The resulting supernatant was collected and retained for subsequent analysis [28].

3.6 Preparation of a standard solution

An initial stock solution of lupeol was prepared by dissolving 2 mg of the standard compound in 2 mL of methanol, yielding a concentration of 1 mg/mL (denoted as Sample A).

Sample A was then subjected to a 1:1 dilution using the appropriate diluent, resulting in a secondary solution with a concentration of 0.5 mg/mL (Sample B).

Sample B was further diluted at a 1:10 ratio with the diluent to obtain a working concentration of 0.05 mg/mL (Sample C). This final dilution (Sample C) was used for quantitative analysis [29].

3.7 Chromatography conditions

Chromatographic analysis was performed using high-performance thin-layer chromatography (HPTLC) on TLC aluminum plates pre-coated with silica gel 60 F_{254} (Merck). Samples and standards were applied as bands onto the plates with a CAMAG Linomat V automatic thin-layer chromatography (TLC) applicator. The plates were prepared in a CAMAG twin-trough glass chamber that had been saturated with the mobile phase vapor for 20 minutes at ambient temperature, with an ascending development distance of 70 mm. For the fingerprinting, identification, and quantification of lupeol, as well as for subsequent elution, a mobile phase of ethyl acetate: formic acid: toluene (4:0.3:6, v/v/v) was employed. After development, the plates were dried and derivatized for visualization using anisaldehyde sulphuric acid reagent (ASR). The derivatized chromatograms were documented under white and UV light at 366 nm, and densitometric scanning was performed using a CAMAG TLC Scanner III controlled by win-CATS software. Furthermore, the prominent bands corresponding to the identified compounds were eluted from the plate using a CAMAG TLC-MS Interface 2 for hyphenated analysis [17, 19, 30, 31, 32].

3.8 Detection and quantitation

The HPTLC plates were placed in a Camag twin through chamber that was previously saturated with the mobile phase consist of Ethyl Acetate: Formic Acid: Toluene (4:0.3:6, v/v/v) for 20 minutes. The plate was developed in a Camag horizontal developing chamber (20 x 10 cm) at room temperature to a height of 7 cm. Ascending method was employed in the creation of Thin Layer Chromatography. Following development, the plates were dried, after which derivatization was conducted with anisaldehyde sulphuric acid reagent using a Camag dip tank. The plate was examined in a UV cabinet using 254 nm, white light, and 366 nm wavelengths. The quantitative evaluation was conducted by screening the plates at 545 nm using Camag TLC scanner III, utilizing Vision-CATS software of Camag. The identity of Lupeol was verified through a spiking investigation. A densitometry HPTLC study was conducted to establish a distinctive fingerprint profile, which may serve as a standard for the quality assessment and regulation of the medications [19, 28, 33].

3.9 Calibration of lupeol

The lupeol concentration was quantified utilizing a calibration curve established with a standard concentration range of 50 to 175 ng/spot. A stock solution of standard lupeol (1 mg/ml) was prepared in methanol. Various volumes of this stock solution 5, 7.5, 10, 12.5, 15, and 17.5 µl of a 0.01 mg/ml dilution were applied to the HPTLC plate to achieve concentrations of 50, 75, 100, 125, 150, and 175 ng/spot, respectively (band width 6 mm, inter-track spacing 12 mm) with automatic sampler. The area under curve for each concentration was graphed against the respective applied quantity of standard lupeol. The linear regression of the standard curve yielded $R^2 = 0.999$. The equation of the linear regression line is $y = 0.00003x - 0.0005$. The regression findings demonstrate a robust linear association within the concentration range of 50–175 ng/spot. The linearity of the calibration graph and the system's adherence to Beer's law were validated by the high correlation coefficient. The low %RSD values for peak areas across all concentration levels (from 0.28% to 1.09%) demonstrate exceptional repeatability. No significant alteration was seen in the gradient of the standard curves (Analysis of variance; $p < 0.05$) [17, 19, 28, 33, 34].

3.10 Validation of the HPTLC Method

3.10.1 Precision

The validation of established analytical method adhered to ICH requirements for precision, reproducibility, and accuracy. The method's instrumental precision, intra-day precision, and inter-day precision were evaluated. Instrumental accuracy was assessed through six replicate applications of the identical Lupeol solution. The intra-day assay precision was evaluated by assessing six duplicate applications of a newly produced standard solution at a uniform

concentration (50 - 175 ng/spot) on the same day. Intermediate accuracy was assessed through the study of six replicate applications of a standard solution at a consistent concentration (50-175 µg/spot) conducted over six distinct days. The consistency of sample application and peak area measurement has been quantified as a percentage coefficient of variation (% CV) [19, 35, 36].

3.10.2 Limit of Detection and Limit of Quantification

To evaluate the limit of detection (LOD) and limit of quantification (LOQ), different concentrations of the standard solutions of lupeol were applied with methanol as a blank and determined based on the signal-to-noise ratio. The LOD was determined at an S/N ratio of 3:1, and the LOQ was determined at an S/N ratio of 10:1 [19, 35, 36]

3.10.3 Specificity

The method's specificity was evaluated by analysing standard Lupeol and its extracts. The presence of Lupeol in the sample was confirmed by comparing the Rf values and spectra of the spot with those of the reference sample. The peak purity of Lupeol was evaluated by comparing the spectra at three distinct levels, i.e., peak start, peak

middle and peak end positions of the spot/ bands [17, 19, 20, 36].

3.10.4 Robustness

The estimation was carried out by varying the selected parameters like mobile phase composition, mobile phase volume, and time of mobile phase saturation within the defined limitation of ±10%, and neither the method's performance nor the outcomes showed any appreciable changes. The findings were presented as the %RSD for data corresponding to each variable condition [19, 37].

3.10.5 Accuracy

The method's accuracy was assessed using recovery studies conducted at 3 different levels (80%, 100%, and 120% addition of Lupeol) with the standard addition technique. Standard concentration of Lupeol (80, 100, 120 µg/spot) were introduced through spiking. The % recovery values and the average % recovery value for lupeol were calculated [19, 37].

3.11 Applicability of Method

3.11.1 System suitability

System suitability tests were conducted to evaluate the adequacy of resolution and repeatability for the analysis. System suitability was evaluated using a freshly prepared standard solution of lupeol (200 ng/spot, six repetitions) under identical chromatographic conditions, followed by scanning and densitogram recording. The peak areas for lupeol and their retention factors were recorded for each concentration of lupeol, and the mean peak area, standard deviation (SD), and coefficient of variation (%CV) were calculated [19, 28, 29, 36].

3.11.2 Estimation of lupeol content in herbal extracts and its isolation

High Performance Thin Layer Chromatography (HPTLC) coupled with a CAMAG TLC SCANNER 3 was utilized to separate, identify, and quantify chemicals in four distinct organic solvents: ethyl acetate, chloroform, ethanol, and methanol [19]. Fingerprint profiles were successfully developed for each extract under white light and at 366 nm after derivatization with anisaldehyde sulphuric reagent (ASR). These profiles revealed a diverse range of phytoconstituents and provided a comprehensive comparative overview of the chemical distribution across the extracts. Subsequently, identification studies were conducted to screen the selected phytomarkers. Among the targeted compounds, only lupeol, a bioactive triterpenoid, was conclusively identified in all three extracts. Its presence was established based on matching Rf values and spectral characteristics with those of the standard, confirming its widespread solubility and extractability across solvents of varying polarities.

Multilevel calibration was employed to quantitatively assess the concentration of lupeol. The ethanol extract had the highest proportion of lupeol, measuring 0.667%. This finding underscores the superior extraction efficiency of ethanol for lupeol, possibly owing to its balanced polarity and compatibility with triterpenoid structures.

Finally, to validate and isolate the observed phytochemical bands, elution was performed using the TLC-MS Interface 2. This technique facilitated the direct extraction and analysis of the separated components from the TLC plate with high precision, enhancing the reliability of compound identification and supporting the fingerprint data.

3.11.3 GC-MS/MS conditions

GCMSMS analysis was conducted utilizing a Thermo Fisher TSQ9000 GC MS/MS (Triple Quad S.No. TSQ92107008) equipment, which was outfitted with an HP 5 MS capillary column with a 5% phenyl polysiloxane as stationary phase (30.0 m × 0.25 mm, film thickness 0.25 µm). Different oven temperatures were evaluated to get optimal separation in the standard lupeol and sample. The instrument was initially set to 60°C for 2 minutes, after which the temperature was elevated at a rate of 10°C per minute till reaching 300°C, followed by an increase to 300°C at a rate of 80°C per minute. The helium carrier gas flow rate was maintained at 1.0 mL/min, the mass spectrometer transfer line temperature was set to 280 °C, and a constant pressure was upheld while the ionization voltage was fixed at 70 eV. Mass spectra were analyzed in scanning mode within the range of 50-1000 m/z. The interpretation of GC-MS/MS spectra was acknowledged and validated using the NIST Mass Spectrometry Data Centre, 2019. The name, Molecular Weight (MW), Retention Time (RT), and structure of the active components were determined. [38, 39].

3.12 Cell Line and Culture Conditions

The human epidermoid carcinoma cell line A431 was procured from the National Centre for Cell Science (NCCS, Pune, India). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; AT149-1L) supplemented with 10% fetal bovine serum (FBS; Himedia, RM10432) and 1% antibiotic-antimycotic solution. Cultures were maintained in a humidified incubator (Heal Force HF-90) at 37°C under 5% CO_2 atmosphere. Cells were routinely sub-cultured to maintain logarithmic growth and were used for experiments during their exponential growth phase. The isolated ethanolic fraction (srbhATETOH) and Lupeol standard were evaluated. Stock solutions were prepared in dimethyl sulfoxide (DMSO) and subsequently diluted in incomplete cell culture medium (without FBS) to achieve the desired working concentrations. The final concentration of DMSO in any well did not exceed 0.1% (v/v), a level confirmed to be non-cytotoxic to the cells in preliminary experiments [40, 41, 42].

3.12.1 Neutral Red Uptake (NRU) Cytotoxicity Assay

Cytotoxicity was assessed using the Neutral Red Uptake (NRU) assay, a well-established method that quantifies cell viability based on the ability of viable cells to incorporate and retain the supravital dye neutral red within lysosomes. A431 cells were inoculated onto 96-well plates at a density of 7,000 cells per well and allowed to adhere for 24 hours. The medium was then replaced with fresh medium containing serially diluted concentrations of the test compounds (srbhATETOH: 0.78–50 µg/mL; Lupeol Standard: 1–1000 µg/mL). After 24 hours of incubation, the treatment medium was removed and cells were incubated with 100 µL of neutral red working solution (40 µg/mL in culture medium)for 1 hour at 37°C. The dye solution was carefully aspirated, cells were washed gently with PBS, and the incorporated dye was eluted using a destaining solution (50% ethanol, 49% deionized water, 1% glacial acetic acid). Absorbance was measured at 550 nm using a microplate reader (iMark, Bio-Rad). Wells containing medium and destaining solution without cells served as blanks. Cell viability was calculated as a percentage relative to vehicle-treated control wells using the formula:

% Cell Viability = (Absorbance of Treated Well / Absorbance of Control Well) × 100

3.12.2 Data and Statistical Analysis

Dose-response curves were generated by plotting the percentage of viable cells against the logarithm of compound concentration. The half-maximal inhibitory concentration (IC_{50}), defined as the compound concentration required to reduce cell viability by 50%, was determined using non-linear regression analysis in GraphPad Prism software (Version 6.0). Data are presented as the mean ± standard error of the mean (SEM) from three independent experiments, each performed in triplicate.

4. RESULTS AND DISCUSSION

4.1 Solvent-Based Extraction Yields of Plant Components

The percentage yield obtained from each solvent extract is summarized in Table 1. Among the four solvents tested, the aqueous extract produced the highest yield (11.2% w/w), indicating superior extraction of water-soluble constituents. This was followed by ethanol (6.3% w/w), which also showed good extraction efficiency. Comparatively lower yields were obtained with ethyl acetate (2.9% w/w) and chloroform (2.5% w/w), suggesting limited solubility of plant components in these less polar solvents.

Table 1 Percentage yield of extract of tubers of Arisaema tortuosum

S. No.	Extract	% Yield (W/W)
1.	Chloroform	2.5%
2.	Ethyl acetate	2.9%
3.	Ethanol	6.3%
4.	Aqueous	11.2%

4.2 Biochemical Profiling of Secondary Metabolites in Plant Extracts

A series of qualitative biochemical assay was conducted to ascertain the presence of principal categories of secondary metabolites in various solvent extracts. The evaluation included the detection of alkaloids, glycosides, flavonoids, diterpenes, phenolic compounds, proteins, carbohydrates, saponins, and tannins by established phytochemical methodologies. The patterns of positive and negative reactions varied across the extracts, reflecting differences in the solubility and distribution of these metabolites. A complete summary of the outcomes for each test and extract is provided in

Table *2*. [27, 43, 44].

Table 2 Result of phytochemical screening of extract of Arisaema tortuosum

S.No.	Constituents	Chloroform extract	Ethyl acetate extract	Ethanol extract	Aqueous Extract
1.	**Alkaloids** Wagner's Test: Hager's Test:	 -ve -ve	 +ve -ve	 +ve +ve	 -ve +ve
2.	**Glycosides** Legal's Test:	 -ve	 -ve	 -ve	 -ve
3.	**Flavonoids** Alkaline Reagent Test: Lead acetate Test:	 -ve +ve	 -ve +ve	 -ve +ve	 -ve +ve
4.	**Diterpenes** Copper acetate Test:	 -ve	 -ve	 +ve	 +ve
5.	**Phenol** Ferric Chloride Test: Folin Ciocalteu Test:	 -ve +ve	 -ve +ve	 +ve +ve	 +ve +ve
6.	**Proteins** Xanthoproteic Test:	 +ve	 -ve	 +ve	 +ve
7.	**Carbohydrate** Fehling's Test: Benedict's Test:	 -ve -ve	 -ve -ve	 +ve +ve	 -ve -ve
8.	**Saponins** Froth Test:	 -ve	 -ve	 +ve	 -ve
9.	**Tannins** Gelatin test:	 +ve	 +ve	 +ve	 +ve

4.3 Result of Estimation of Total Flavonoids Content and Total Phenolic Content

Total flavonoid content and phenolic content expressed as quercetin equivalent and gallic acid equivalent, respectively, arrived at from the standard calibration line Eqns. $y = 0.036x + 0.015$; $R^2 = 0.999$ and $y = 0.014x - 0.013$; $R^2 = 0.999$ represented in Figure 3.

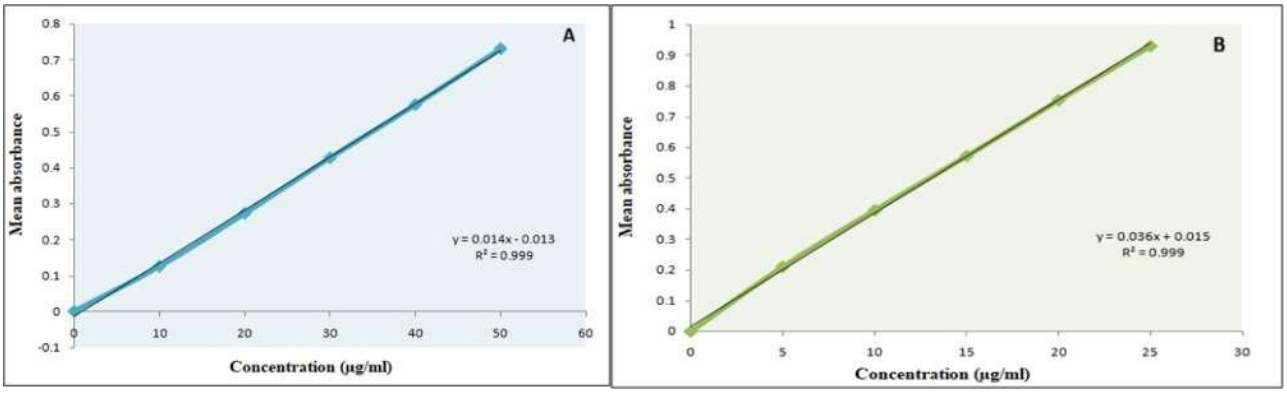

Figure 3 Calibration curve of Gallic acid and Quercetin.

The Ethanolic extract of Arisaema Tortuosum contained a higher amount of total flavonoid content (0.805mg/100 mg) and total phenolic content (0.878mg/100 mg) as compare to other extract as shown in Figure 3.

Table 3 Total Phenolic and Total flavonoid content of different extract of Arisaema Tortuosum

S.NO	Extract	Total phenol content (mg/100 mg)	Total flavonoids content (mg/100 mg)
1.	Chloroform	0.392	0.552
2.	Ethyl acetate	0.485	0.422
3.	Ethanol	0.878	0.805
4.	Aqueous	0.671	0.594

4.4 FINGERPRINT AND CHROMATOGRAPHY

HPTLC analysis were performed on silica gel 60 f-254, 20 x10 cm HPTLC plates (Merck, Germany- 5642) with Ethyl acetate: formic acid: toluene (4:0.3:6 v/v/v) for fingerprinting and lupeol quantification, Derivatization: Anisaldehyde-sulphuric acid (ASR) was used for band visualization. Fresh tubers of *Arisaema tortuosum* (AT) were processed into powder and extracted using four solvents: ethyl acetate, ethanol, chloroform, and methanol, the Rf value was about 0.67.

The extracts of A. tortuosum tubers and lupeol standard solutions (5.0 µl of each concentration at 0.05 mg/mL) were applied to the HPTLC plates using a CAMAG-Linomat IV automated band applicator with a 100 mL syringe. The band length is of 8 mm, an application rate of 10 sec/mL, a distance of 4 mm between bands, a distance of 1.5 cm from the plate's side edge and a distance of 2 cm from the bottom of the plate. Subsequent to development, the plates were dried on CAMAG TLC Plate Heater. A CAMAG TLC Scanner 3 was employed for densitometric quantification of the bands utilizing Vision CATS software. The operational parameters of the scanner were are- Mode: absorption/reflection; slit dimensions: 5 x 0.1 mm; scanning rate: 20 mm/s; monochromatic bandwidth: 20 nm at optimum wavelengths of 254 nm, 366 nm and within the visible spectrum [17, 28, 45, 46].

4.4.1 Preparation of Sample:

For extraction of bioactive compounds from *Arisaema tortuosum* (AT), powdered plant material (300 mg) was separately dissolved in four different organic solvents: ethyl acetate, chloroform, ethanol, and water. Each mixture was sonicated for fifteen minutes to enhance solubility, followed by centrifugation at 3000 rpm for 10 minutes to filter out the insoluble residue. The resulting supernatant was carefully collected and transferred into vials for subsequent analytical applications.

4.4.2 Standards Preparation:

An initial stock solution of Lupeol was prepared by dissolving 2 mg of the standard compound in 2 mL of methanol, yielding a concentration of 1 mg/mL (denoted as Sample A).

a. Sample A was then subjected to a 1:1 dilution using the appropriate diluent, resulting in a secondary solution with a concentration of 0.5 mg/mL (Sample B).
b. Further dilution of Sample B was performed at a 1:10 ratio with the diluent to obtain a working concentration of 0.05 mg/mL (Sample C). This final dilution (Sample C) was utilized for quantitative analysis.

4.4.3. Identification

The HPTLC analysis of *Arisaema tortuosum* tuber extracts successfully established distinct chromatographic fingerprints under various visualization conditions. The optimized mobile phase ethyl acetate:formic acid: toluene (4:0.3:6 v/v/v) provided excellent separation of phytoconstituents across all solvent extracts. As evidenced in Figure 4, the derivatized plates revealed complex banding patterns with varying intensities and colors, indicative of the diverse phytochemical composition.

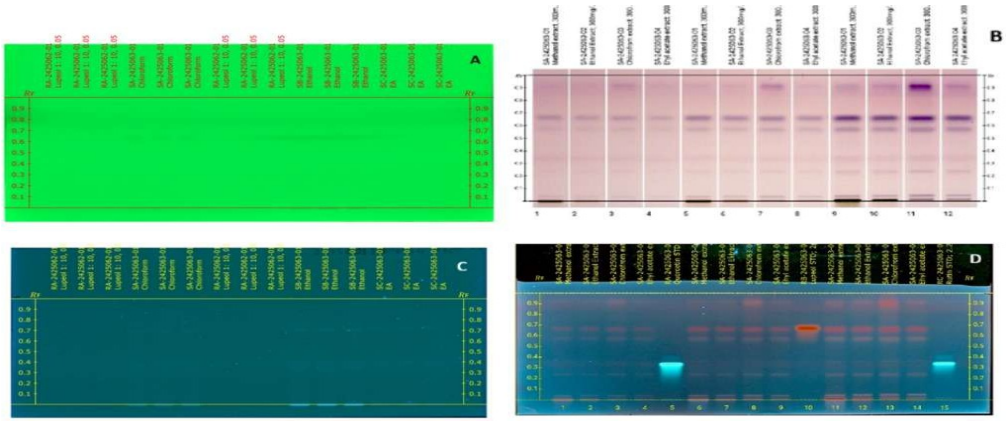

Figure 4 HPTLC profile of Arsiema tortuosum under different conditions. (A) Plate visualized at 254 nm before derivatization.(B) Plate visualized under white light after derivatization with Anisaldehyde Sulphuric acid reagent. (C) Plate visualized at 366 nm before derivatization. (D) Plate visualized at 366 nm after derivatization

The ethanol extract consistently demonstrated more intense and well-defined bands, suggesting superior extraction efficiency for multiple compounds. The identity of lupeol was conclusively established through co-chromatography with the authentic standard, showing perfect alignment at Rf 0.67 across all visualization modes. The specificity of detection was further confirmed by spectral analysis, which showed identical absorption patterns between the standard and sample bands.

4.5 VALIDATION OF HPTLC METHOD

The proposed HPTLC method demonstrated excellent performance characteristics and is validated as per ICH guidelines. The linearity study revealed a strong correlation between concentration and peak area across the range of 50-175 µg/spot, with a correlation coefficient (R^2) of 0.999 as shown in Table 4 [19]. The regression equation was determined as Y = 0.00002919X - 0.00028255, confirming excellent linear relationship within the tested range [36, 35].

Table 4 Performance parameters of the proposed TLC densitometric technique for the measurement of lupeol

Parameter	**Method (Lupeol)**		**Acceptance criteria (maximum acceptable)**
Selectivity	Selective		--
Specificity	Specific		No interference observed
Linear range (µg/spot)	50 µg to 175 µg		Linearity, accuracy and precision over the range
Correlation coefficient (r^2)	0.999		Within 0.9–1.1
Linear regression equation Y = mX + c	Y=0.00002919X-0.00028255		-
LOD (µg /spot)	1.22 µg /spot		-
LOQ (µg /spot)	3.70 µg /spot		-
% Recovery	99.92%-100.05%		Within 90–110%
Repeatability (%RSD, n = 6)	0.15-0.21		%RSD ≤ 2%
Precision (%CV)	Intraday	Interday	%CV ≤2%
	0.67-1.03	0.60-1.20	

Method precision was rigorously evaluated through intra-day and inter-day studies, with results demonstrating excellent reproducibility as detailed in Table 5 [19]. The percentage coefficient of variation values remained below 2% across all concentration levels, confirming the method's reliability for quantitative analysis.

Table 5 Intraday precision and interday precision of HPTLC method

Concentration of standards (µg/spot)	Intraday precision	Inter day precision
	SD in area	%CV
50	0.0000125	1.03
75	0.0000145	0.75
100	0.0000175	0.67

The accuracy of the method, as determined through recovery studies using the standard addition method and presented in Table 6. [19], yielded excellent results with percentage recovery ranging from 99.92% to 100.05%. These values, well within the acceptable limits of 90-110%, confirm the method's accuracy and absence of matrix interference. [37]

Table 6 Recovery analyses of Lupeol at 80%, 100%, and 120% additions with the proposed TLC densitometric method

Concentration of standards (µg/spot)		Area spotted		Total area (sample + standard)	Total area obtained	% Recovery
Sample content	Spiked amount	Mean Sample area	Mean Spiked area			
100	80	0.002629	0.002103466	0.004732799	0.004729115	99.92%
100	100	0.002629	0.002629333	0.005258666	0.005261086	100.05%
100	120	0.002629	0.003155200	0.005784533	0.005782490	99.96%

The method exhibited adequate sensitivity with limit of detection (LOD) and limit of quantification (LOQ) values of 1.22 and 3.70 µg/spot, respectively, as documented in Table 4. The robustness of the method was confirmed through deliberate variations in chromatographic parameters, which did not significantly affect the analytical outcomes.

4.5.1 Quantitative Estimation of Lupeol

The validated HPTLC method was successfully applied for the quantification of lupeol in different solvent extracts of *A. tortuosum* tubers. The densitometric analysis, as shown in Figure 5 revealed distinct peaks corresponding to lupeol at Rf 0.67 in all extracts.

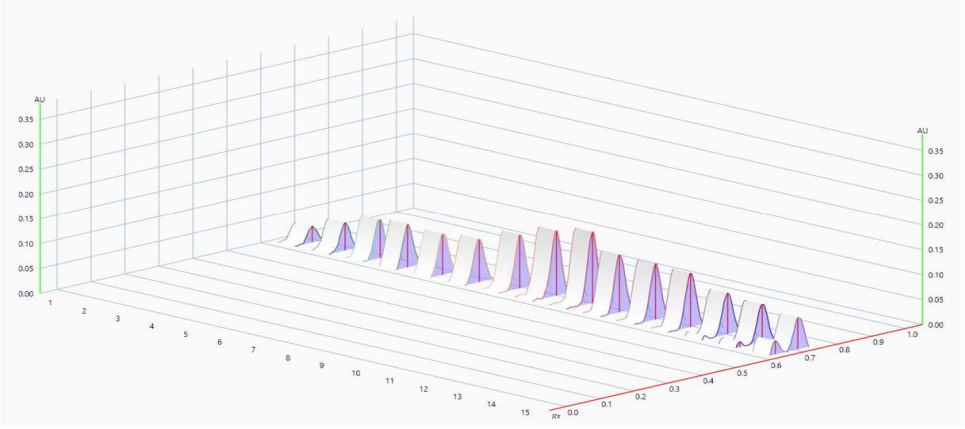

Figure 5 Densitometric chromatogram of lupeol and extract of A. tortuosum (tuber) at 540 nm (3D view).

The ethanol extract contained the highest concentration of lupeol at 0.667% w/w, followed by chloroform extract (0.525% w/w), ethyl acetate extract (0.491% w/w) and aqueous extract (0.074% w/w) as represented in Table 7. The densitometric profile in Figure 6. clearly illustrates the resolution and peak purity of lupeol in the sample tracks.

Table 7 Amount of Lupeol present in different extract of Arisaema tortuosum

S.No	Sample Extract	Result	Percentage
1	Ethanol	1.332 mg in 200.0 mg	0.667%
2	Chloroform	1.050 mg in 200.0 mg	0.525%
3	Ethyl Acetate	982.5 µg in 200.0 mg	0.491%
4	Aqueous	149.12 µg in 200.0 mg	0.074%

Comparative analysis of the densitometric chromatograms in Figure 6 further confirms the superior extraction efficiency of ethanol for lupeol, as evidenced by the higher peak intensity in the ethanol extract track compared to other solvent extracts.

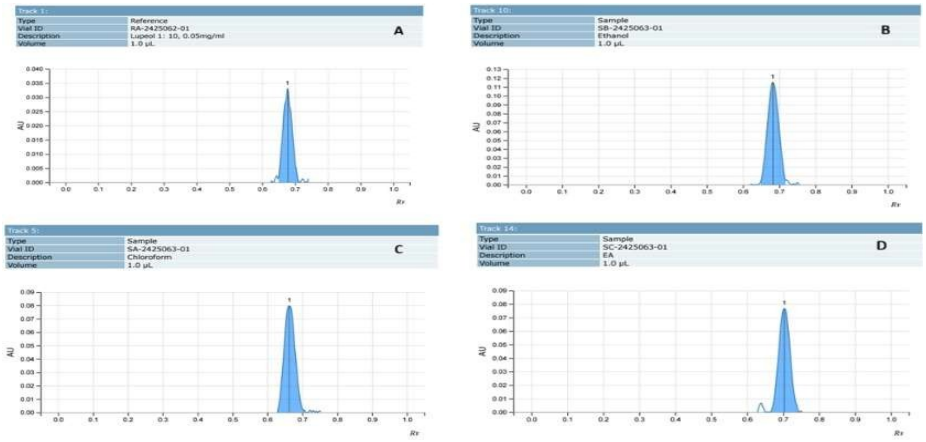

Figure 6 Comparative HPTLC densitometric profiles of standard lupeol and various extracts of A. tortuosum. Track A corresponds to the lupeol standard, while tracks B, C, and D represent the ethanol, chloroform, and ethyl acetate extracts, respectively demonstrating the presence of lupeol

4.5.2. Isolation of Lupeol from *Arisaema tortuosum*

The HPTLC-assisted fractionation of the plant extract yielded a discrete band corresponding to the lupeol standard, which was subsequently isolated using the HPTLC interface-2 system as mentioned in Figure 7.

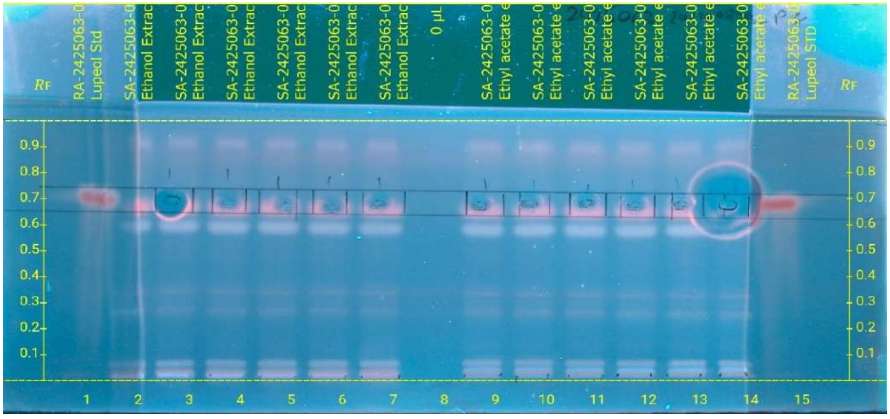

Figure 7 Image after TLC-MS Interface and Derivatization with Anisaldehyde Sulphuric acid reagent

4.6 Characterization by Mass Spectrometry

The isolated fraction obtain from the HPTLC interface-2 system was analysed by GC-MS/MS to confirm the identity of the compound. Chromatographic analysis revealed a sharp, well- resolved peak in the plant-derived sample with a retention time (RT) of 31.85 minutes (Figure 8 A). This aligned almost identically with the RT of 31.86 minutes observed for the authentic lupeol standard (Figure 8B), demonstrating excellent chromatographic concordance under the employed analytical conditions.

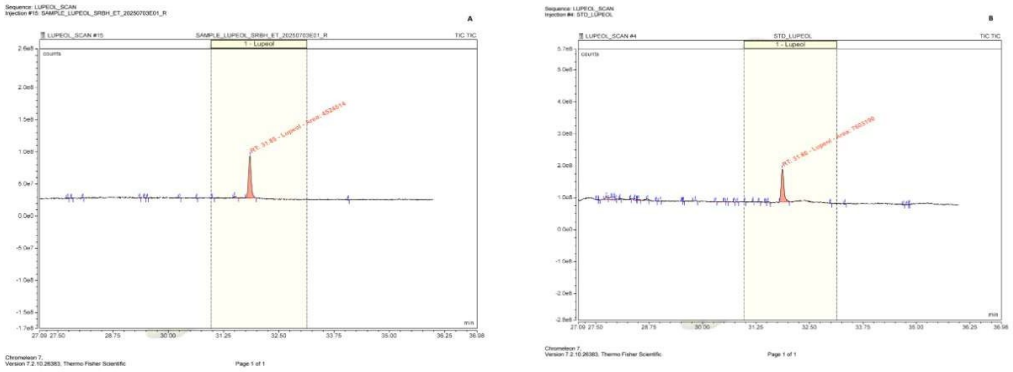

Figure 8 Chromatographic Profiling (GC) of Lupeol: Comparative Analysis of Plant Sample and

Authentic Standard

Mass spectrometric examination provided definitive structural confirmation. The plant isolate exhibited a prominent molecular ion peak at m/z 426 (Figure 9A), corresponding to the molecular formula $C_{30}H_{50}O$. This mass spectral signature was indistinguishable from that of the reference lupeol (Figure 9B). Furthermore, the fragmentation pattern of the isolated compound was meticulously compared with the NIST 2019 mass spectral library. The observed fragments, including key ions at m/z 189, 207, and 218, which are characteristic of the lupane skeleton, showed a high degree of matching with the library entry for lupeol (NIST: 513124). The forward and reverse match factors for the sample were 792 and 876, respectively, with a high probability score of 32.3%, providing robust statistical support for the identification. These spectral data, combined with the chromatographic co-elution evidence, conclusively identify the isolated compound as lupeol.

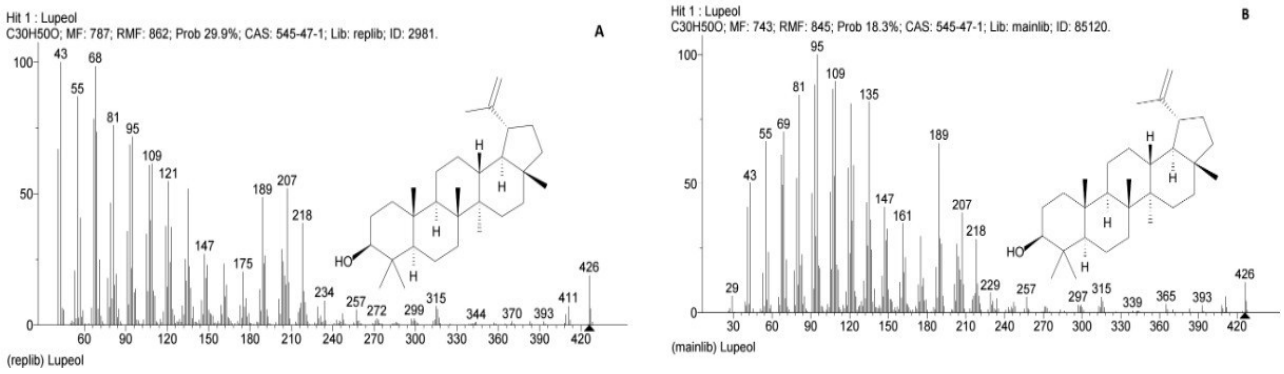

Figure 9 GC-MS-MS Mass Spectra Analysis: Structural Confirmation of Lupeol in Plant Extract and Standard

4.7 Cytotoxic Activity of Isolated Compounds Against A431 Cells

The NRU assay was employed to evaluate the cytotoxic potential of *Arisaema tortuosum* tuber extract (Sample) and pure lupeol (Lupeol) against human epidermoid carcinoma A431 cells. Both samples exhibited concentration-dependent reductions in cell viability following 24-hour exposure. Both compounds induced a concentration-dependent decrease in cell viability, as illustrated in Figure 10.

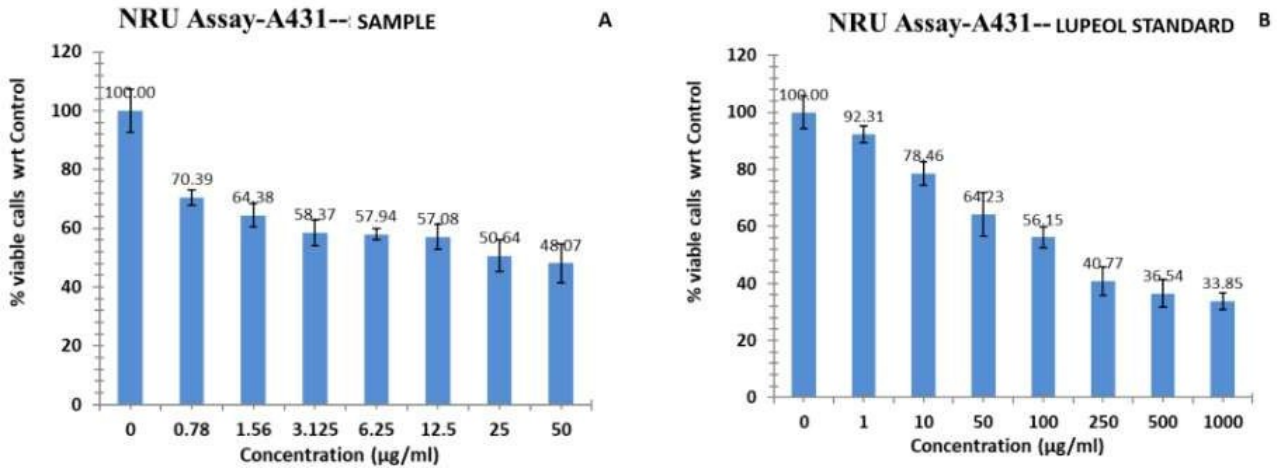

Figure 10 Dose-response curves revealing superior cytotoxicity of A. tortuosum extract. The ethanolic fraction (Sample, IC_{50} 13.49 ± 0.199 µg/mL) induced a sharp decline in A431 cell viability, in stark contrast to the minimal activity of the Lupeol Standard compound (IC_{50} 168 ± 0.127 µg/mL).

The crude extract sample demonstrated potent activity, with cell viability declining sharply across the tested range. The IC_{50} value was calculated to be 13.49 ± 0.199 µg/mL. In contrast, purified Lupeol Standard showed a more gradual decrease in viability, yielding a significantly higher IC_{50} value of 168 ± 0.127 µg/mL as mentioned in Table 8. These results indicate that the tuber extract possesses markedly greater cytotoxic potency against A431 cells compared to the isolated lupeol compound.

Table 8 Cytotoxic activity of isolated compounds against A431 cells as determined by the NRU assay.

S.No.	Compound	IC_{50} Value (Mean ± SEM)
1.	Plant extract sample (Code- srbhAT ETOH)	13.49 ±0.199 µg/mL
2.	Lupeol Standard (Code- Lup-Std)	168 ±0.127 µg/mL

These results unequivocally identify the ethanol extract of *Arisaema tortuosum* tubers as the more promising cytotoxic agent, demonstrating approximately 12.5-fold greater potency than the purified Lupeol Standard in this *in vitro* model of human epidermoid carcinoma (A431 cells).

CONCLUSION

This comprehensive investigation into the tuber extracts of *Arisaema tortuosum* (Wall.) Schott has elucidated a rich and varied phytochemical profile, the composition of which is markedly influenced by solvent polarity. The significant range in extractive yields from 2.5% using non- polar chloroform to 11.2% with a polar aqueous solvent provides a clear initial indicator of the plant matrix's diverse chemical constituency. This polarity-based gradient suggests a particular abundance of medium- to high-polarity bioactive compounds, a premise strongly reinforced by subsequent phytochemical screening. The detection of a wide array of secondary metabolites, including alkaloids, phenols, diterpenes, and tannins, offers a tangible chemical rationale for the ethnomedicinal uses historically associated with *Arisaema* species. Of particular note, the ethanol extract emerged as an exceptionally efficient and balanced solvent, proficient in extracting a broad spectrum of both polar and moderately non-polar bioactive constituents, as evidenced by its robust yield and the most comprehensive phytochemical signature.

A central methodological contribution of this work is the development and rigorous validation of a novel HPTLC-densitometric protocol for the quantification of lupeol, conducted in full compliance with ICH Q2(R1) guidelines. The method demonstrated excellent linearity (R^2 = 0.999), high precision with intra- and inter-day coefficients of variation below 2%, and accuracy with recovery rates approximating 100%, establishing it as a dependable tool for the standardization of *A. tortuosum* preparations. Its high sensitivity, indicated by low limits of detection and quantification, makes it suitable for quantifying lupeol even when present as a minor component.

The application of this validated analytical method enabled the precise quantification of lupeol across the different extracts. The ethanol extract was found to contain the highest concentration (0.667% w/w), a chemically logical finding given lupeol's intermediate polarity. Unambiguous identification was achieved through orthogonal techniques, including a distinct retardation factor (Rf = 0.67) matching the authentic standard, confirmation via co-chromatography, and conclusive structural elucidation using HPTLC coupled with GC-MS/MS. The GC-MS/MS analysis provided definitive proof through near-identical retention times and a diagnostic mass spectral fingerprint consistent with the lupane skeleton.

These analytical findings gain substantial biological relevance when correlated with the results of the cytotoxicity evaluation. The *in vitro* assessment against the human epidermoid carcinoma A431 cell line yielded compelling data. The ethanolic extract of *A. tortuosum* demonstrated potent, dose-dependent antiproliferative activity with a calculated IC_{50} value of 13.49 ± 0.199 µg/mL. In stark contrast, the lupeol standard exhibited a significantly weaker cytotoxic effect, with an IC_{50} of 168 ± 0.127 µg/mL. This pronounced difference in potency where the crude ethanol extract is approximately 12.5-fold more effective strongly indicates that the observed anticancer activity is not mediated by lupeol in isolation. Instead, it points towards a likely synergistic interplay between lupeol and the other abundant bioactive compounds, such as phenolics and flavonoids, quantified within the complex extract matrix. The superior activity of the ethanol fraction rationally validates the prioritization suggested by its exemplary phytochemical profile.

In conclusion, this research effectively bridges the long-standing ethnobotanical use of *Arisaema tortuosum* with modern scientific validation through a combined analytical and biological approach. A rigorously tested HPTLC method was established for reliable quality control, and a key bioactive compound, lupeol, was precisely characterized. Most importantly, the study demonstrates a clear connection between the plant's complex phytochemical makeup and its measurable anticancer effect against epidermoid carcinoma cells.

The validated analytical protocol and the compelling bioactivity data together create a strong scientific basis for developing standardized herbal formulations. The consistent chemical profile of the potent ethanol extract, in particular, allows for the production of quality-controlled preparations with reliable therapeutic effects. These findings enable a direct transition from laboratory research to pre-formulation science, where this well-defined extract can be incorporated into advanced delivery systems including nano formulations, oral capsules, or topical applications to improve its stability, absorption, and clinical potential for dermatological or oncological use.

Consequently, the ethanol extract of *Arisaema tortuosum* stands out as a highly promising candidate for future drug development. This work sets a firm groundwork for subsequent studies aimed at isolating the most active fractions through bioassay-guided processes, deciphering the exact molecular mechanisms involved, and understanding the synergistic interactions between the various plant constituents. Together, these efforts will be crucial for fully realizing the therapeutic promise of this significant medicinal plant.

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Declaration of Competing Interest
No conflict of interest is declared by the authors.

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